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NOVEL BLOOD MARKERS IN PSYCHOSIS

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NOVEL BLOOD MARKERS IN PSYCHOSIS

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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This thesis is dedicated

To persons affected by psychotic disorders past and present,

To health care practitioners, especially those concerned with psychiatric illnesses and cardiovascular diseases,

To exercise physiologists, physiotherapists and physical trainers.

ABSTRACT

Psychotic disorders such as schizophrenia maintain a 1% point prevalence in society at large. They cause much suffering to the patients and exact grave costs on society. There is an urgent need for better diagnosis and treatment of psychotic disorders. Patients with psychotic disorders are primarily treated for deteriorating psychiatric symptoms and often somatic comorbidities are overlooked. Accelerated ageing and metabolic comorbidities such as adiposity, diabetes and cardiovascular disease are common in psychosis patients, with often 15-20 years shortened life expectancy. The main pathophysiological elements of psychotic disorders and schizophrenia remain elusive. There is increasing evidence to suggest that psychotic disorders such as schizophrenia are in fact a group of related disorders advocating a more individualized evidence-based treatment than that currently available. Biomarkers are by definition useful tools for describing individual patient centered disease states or selecting treatment approaches. The main aim of this thesis was to investigate putative blood biomarkers which describe the previously reported ongoing pathological processes inflammation, mitochondrial dysfunction and metabolic disturbances. In **paper I** we assessed, mitochondrial DNA (mtDNA), a proxy for mitochondrial dysfunction in patients of psychotic disorder. We additionally investigated the effect of anti-psychotic drug treatment on the mtDNA copy number of neuro-epithelial stem cell derived human neurons. In **paper II and III,** we explored plasma levels of GDF-15, an anti-inflammation marker gaining traction in the field of cardiovascular disease, in the context of psychotic disorders. In **paper IV** we report the initial findings of a larger study of inflammation in first episode psychosis (FEP) patients recruited for exercise intervention. We investigated a pre-selected group of cytokines, ligands and receptors in patients with FEP.

The major findings from this thesis work includes 1) We were the first to report elevated plasma GDF-15 levels in patients with psychotic disorders compared to healthy age and gender matched controls. 2) We detected that GDF-15 robustly associated with aging and levels of established analyte biomarkers for cardiovascular disease, while not with the acute inflammation marker C-reactive protein. 3) Treatment with clozapine and risperidone was associated with a depletion of whole blood and neuronal mtDNA. 4) In those not treated with clozapine or risperidone, the mtDNA copy number was reduced with age and with more severe psychosis. 5) Most FEP patients, 70%, had markedly elevated plasma fractalkine levels, while the rest had low healthy control levels, reflecting an on/off pattern. FEP patients could be divided into four immunologically distinct groups categorized by how fractalkine levels changed over 12 weeks physical exercise. One group likely represents patients with milder psychosis. The present thesis provides findings from pre-clinical and basic research with a potential to support the development of better clinical therapy for patients of psychotic disorders.

Keywords: mitochondrial DNA, ageing, inflammation, psychosis, anti-psychotic drugs, schizophrenia, exercise, fractalkine, GDF-15

LIST OF PUBLICATIONS/MANUSCRIPTS

I. **Kumar P**, Efstathopoulos P, Millischer V, Olsson E, Bin Wei Y, Brüstle O, Schalling M, Villaescusa JC, Ösby U, Lavebratt C.
Mitochondrial DNA copy number is associated with psychosis severity and anti-psychotic treatment.
Scientific Reports. 2018 August Vol. 8, Article no.: 12743 doi: 10.1038/s41598-018-31122-0

II. **Kumar P**, Millischer V, Villaescusa JC, Nilsson IAK, Östenson CG, Schalling M, Ösby U, Lavebratt C.
Plasma GDF15 level is elevated in psychosis and inversely correlated with severity.
Scientific Reports. 2017 August Vol. 7, Article no.: 7906 doi: 10.1038/s41598-017-07503-2

III. **Kumar P**, Olsson E, Forsberg S, Hukic DS, Westman J, Schalling M, Edman G, Eriksson SV, Ösby U, Lavebratt C.
Cardiac biomarkers for patients with psychotic disorder in Sweden.
Manuscript

IV. **Kumar P**, Lambden B, Yacaman-Mendez D, Ekblom Ö, Skott M, Fogdell Hahn A, Forsell Y, and Lavebratt C.
Elevated plasma Fractalkine levels in first episode psychosis patients is normalized by exercise intervention.
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

ADHD	Attention Deficit Hyperactivity Disorder
ASD	Autism Spectrum of Disorders
BD I	Bipolar type I
BD II	Bipolar type II
BMI	Body Mass Index
CHD	Coronary Heart Disease
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CVD	Cardiovascular Disease
DBP	Diastolic Blood Pressure
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders-Fourth Edition
ELISA	Enzyme-linked Immunosorbent Assay
FEP	First Episode Psychosis
Fwd	Forward
HDL	High Density Lipoprotein
Hs-cTNT	High-sensitive cardiac troponin T
Hs-CRP	High-sensitivity C-reactive protein
IFNγ	Interferon gamma
IL	Interleukin
ICD-10	International Statistical Classification of Diseases and Related Health Problems - Tenth Revision
IMT	Intima-Media Thickening
LDL	Low Density Lipoprotein
NESC	Neuro-epithelial Stem Cell
NMDA	N-methyl-D-aspartate
NT-ProBNP	N-terminal prohormone of brain natriuretic peptide
NFKB	Nuclear Factor-Kappa B
PBMC	Peripheral Blood Mononuclear Cell
RMA	Repeated Measures ANOVA
ROS	Reactive Oxidative Species
Rv	Reverse
SBP	Systolic Blood Pressure
SZ	Schizophrenia
TRAIL	TNF-Related Apoptosis-Inducing Ligand (TRAIL)
TNF-α	Tumor Necrosis Factor Alpha
VEGF	Vascular Endothelial Growth Factor

1 INTRODUCTION

1.1 PSYCHOSIS AND SCHIZOPHRENIA

1.1.1 Symptoms and Diagnosis

Psychosis can be defined as a dissociation from reality based on irregular thoughts and impediment of cognitive function. Two examples of psychiatric diagnoses amongst many disorders which present psychotic symptoms are bipolar disorder Type I (BD I) and schizophrenia (SZ). To obtain a diagnosis of SZ, at least 2 main psychotic symptoms should be strongly prevalent in an individual over the course of a month and mental disturbances should be present over a period of 6 months. Based on the criteria of the American Psychiatric Association's fourth revision of the Diagnostic and Statistical Manual of Mental Disorders [DSM-IV;[1]] or the World Health Organization's 10th edition of the International Statistical Classification of Diseases and Related Health Problems [ICD-10;[2]] these psychotic symptoms include, hallucinations, delusional beliefs, catatonic behavior, negative affect and irregular speech. Generally, a trained psychiatrist considering the cultural norms of the patient makes a diagnosis using the above-mentioned instruments, using her observations and account of responses to questionnaires over a period of time.

1.1.2 Etiology of Schizophrenia a psychotic disorder

SZ is a debilitating psychiatric disorder with a prevalence estimated at 1% of the world population [3]. It is a syndrome that causes a range of detrimental symptoms to afflicted individuals such as loss of cognitive function, social and occupational functioning and increased risks of lifetime morbidity and mortality [4]. The cause of SZ is far from certain and likely heterogeneous. However, strong links have been established to the genetic makeup of an individual, through twin concordance studies, in particular, showing that the polygenic model best explains empirical findings [5]. A Finnish adoptive family study found that although there was evidence for a genetic hypothesis in SZ, biologically predisposed offspring in disturbed or stressful adoptive families were most vulnerable to the syndrome, thus illustrating the effect of the environment on disease etiology [6]. The mechanism by which genetic traits combine with environmental effects to give rise to SZ is poorly understood however there is evidence that malnutrition before birth, exposure to certain virus prenatally, obstetric complications, heavy cannabinoid use and challenging socio-economic factors are some of the environmental factors that have been linked with SZ [7-11]. Increasingly, regulatory elements of gene expression such as non-coding RNA [12] and epigenetic mechanisms [13] have been implicated in SZ etiology. The action of dopamine antagonists in ameliorating SZ positive symptoms and hypofunction of glutamatergic signaling via NMDA receptors implicated in both positive and negative symptoms of SZ has led to hypotheses that the dopaminergic and glutamatergic pathways, when disrupted lead to the constellation of symptoms described as SZ[14,15]. Complimentary to these twin pillars of SZ etiology, there is a developing theory that explains the neuropathology of SZ, through inflammation. Here the theory posits that genetic anomalies in the presence of environmental insults manifest as chronic inflammation and alterations in the cytokine profiles of afflicted individuals, which in early life gives rise to SZ [16].

1.2 THE IMMUNE SYSTEM AND THE INFLAMMATORY RESPONSE

The human body benefits from a complex repertoire of molecular and cellular mediators of defense mechanisms against invading pathogens. An activation of host defense system is often accompanied by inflammation, a humoral and cellular mediated response to pathogenic material, irritants, toxins and traumatic breach of membranes. The purpose of the initial inflammatory response is to eliminate the invading pathogen or irritant and begin the process of repair. The acute inflammatory response and ongoing inflammation is highly regulated by the human body as chronic or unchecked inflammation is associated with many somatic disorders[17]. In SZ, there is a growing consensus for inflammation to be elevated above physiological levels. The reasons for this are manifold, including unchecked chronic inflammation likely caused by an interaction between the host's genetic background, and exposure to foreign pathogens, or toxic by-products of lifestyle factors, such as smoking or alcohol abuse. In a meta-analysis, drug-naïve first-episode psychosis (FEP) patients were found to have higher levels of interleukin-1β (IL-1β), IL-6, tumor necrosis factor alpha (TNF-α) [18] and cardio-metabolic disturbances [19-21]. C-reactive protein (CRP), a canonical circulating indicator of inflammation has also been found to be elevated in SZ [22]. Peripheral inflammation has been implicated in the activation of inflammatory networks in the central nervous system (CNS) [23]. And, conversely, immune activation in CNS, in particular chronic microglial activation, is proposed to be a source of inflammation in peripheral blood; perinatally primed subsets of microglial cells have been suggested to release pro-inflammatory cytokines to the periphery[24]. Correspondingly, increased levels of pro-inflammatory cytokines and kynurenine metabolites were found in the cerebrospinal fluid and in the serum of chronic SZ patients in independent investigations [25,26]. In this thesis, mediators of inflammation based on existing literature, were investigated in blood samples from patients with psychosis[27-33]. The mediators for inflammation were noted for their attributes of neurotrophicity and previous reports of alterations in cohorts of psychotic disorders (Figure 1).

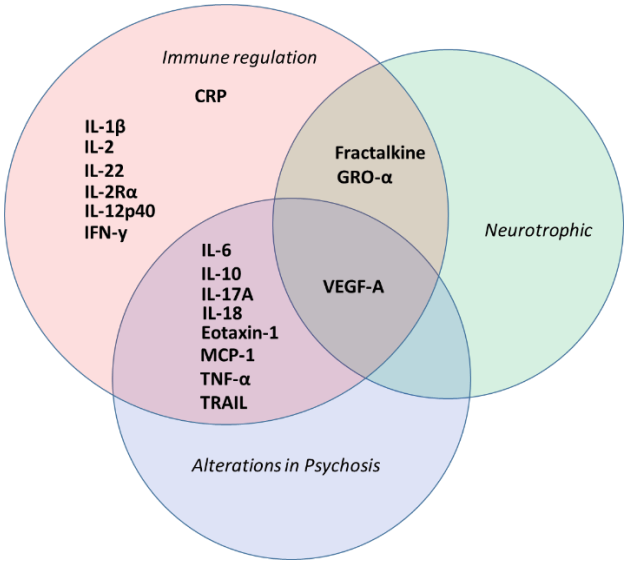

Figure 1: Mediators of inflammation were selected based on having neurotrophic characteristics, having been reported in the literature as being altered in patient material of psychotic disorders and being involved in immune regulation. Individual hypotheses were constructed for CRP, IL-1β, IL-2, IL-22, IL-2Rα, IL-12p40 and IFNγ that they would be dysregulated in patients of psychotic disorders. In addition, IL-6, IL-10, IL-17A, IL-18, Eotaxin-1, MCP-1, TNFα, TRAIL were investigated to confirm previous reports of alterations in patients of psychotic disorder as well as for their defined roles in immune regulation. Little is known on the alterations of fractalkine and GRO-α in the context of psychotic disorders, while VEGF-A is at the intersection of all 3 research spaces of this study.

1.3 MITOCHONDRIAL DYSFUNCTION

The mitochondrion is a highly dynamic subcellular organelle involved in the production of ATP which powers the cell. Mitochondrial dysfunction through environmental stressors[34] or hereditary factors[35] have been implicated in various somatic and neurodegenerative disorders [25,36,37] and in small patient groups of bipolar disorder (BD) and SZ [38,39]. Mitochondrial dysfunction and associated by-products, such as Reactive Oxygen Species (ROS) are implicated in caspase activation[40], inflammasome recruitment[41] and the activation of downstream cytokines and inflammatory mediators[42] which can ultimately lead to increased CNS localized apoptosis and neurodegeneration. Mitochondrial dysfunction is associated with an increased presence of ROS superoxide anion (O_2^-). O_2^- is responsible for the activation of redox-sensitive transcription factor and primary tissue inflammation regulator nuclear factor-κB (NF-κB) which in turn causes an increase in the expression of cytokines, chemokines, eicosanoids, inducible nitric oxide synthase (iNOS) and adhesion molecules[43,44](Figure 2). Therefore, the mitochondrial health of SZ patients has been subject to increasing scrutiny with the aim to characterize mitochondrial abnormalities in SZ and BPD patients versus normal healthy controls. In the striatum of SZ subjects, treatment responsive patients had 40% decrease in synaptic mitochondrial density in the caudate nuclease and putamen compared to healthy controls[45]. Mitochondria were noticeably smaller in the prefrontal cortex (PFC) of BPD patients compared to healthy controls[46]. Oligodendrocytes in the PFC of SZ patients showed remarkable reductions in mitochondrial numbers and volume[47]. In a comparative analysis of white blood cell mitochondria between SZ and healthy controls it was found that SZ patients had fewer mitochondria which showed swelling and fragmentation of the cristae[48]. The hyper-oxidative states and chronic inflammation brought about by psychiatric illnesses create a closed loop between mitochondrial dysfunction and inflammation creating a vicious cycle of events[44] which may explain the accelerated ageing and multifaceted co-morbidities experienced by SZ patients [49]. One method of investigating mitochondrial functionality, which can be applied to cohorts of psychotic disorders, was reported by Lee and Wei[50], where mtDNA copy number is investigated as a proxy for mitochondrial biogenesis and mitochondrial functionality. Using a real time polymerase based method of quantifying DNA, mtDNA copy number from patient leukocytes can be measured in patients in a non-invasive way[51].

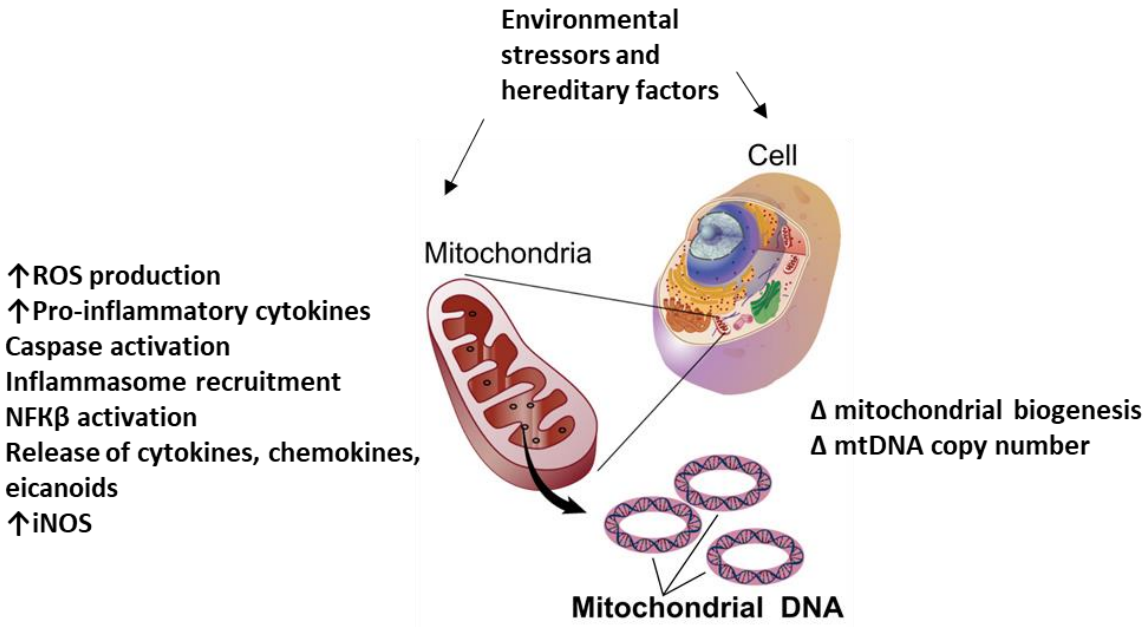

Figure 2: The mitochondria is a highly dynamic organelle which interchangeably exists as a discrete organelle and a part of a sub-cellular vesicular network, depending on cellular bioenergetics, stress and exposure. Multiple copies of the mitochondrial DNA exist within the mitochondrion, which is replicated upon mitochondrial fission. Measuring the copies of mtDNA has been used as a sensitive measure of mitochondrial health and functionality in the context of psychotic disorders. This method has been refined and used for the purpose of **Paper I** of this thesis.

1.4 GDF-15

Apart from directly measuring mitochondrial morphology and volume or proxy measurements such as mtDNA content, a group of researchers have linked mitochondrial dysfunction and diseases to the upregulation of an all-cause mortality marker called Growth differentiation factor-15[52,53]. GDF-15 has been characterized to be a novel biomarker for diagnosing mitochondrial disorders and tracking the treatment outcome of pyruvate therapy in mitochondrial disease [52]. GDF-15 is a member of the transforming growth factor- β (TGF-β) superfamily of cytokines, which is upregulated in response to injury, with defined roles in regulating inflammation and apoptosis[54]. It is suggested to have an overall anti-inflammatory effect and is elevated in cancer, cardiovascular disease (CVD) and obesity, as a compensatory mechanism [33,55]. In CNS GDF-15 is upregulated in response to injury such as cold-induced lesions *in vivo* with an additional role as a neurotrophic factor [56]. Strelau et al have reported that GDF-15 is neurotrophic and neuroprotective in neurons *in vitro* and *in vivo* where intoxicated dopaminergic neurons of the 6-hydroxydopamine rat model of parkinsonism are rescued, thus abolishing an abnormal turning behavior [57]. Interestingly, a proteomics investigation for biomarker development conducted by Frye et al., has returned GDF-15 as a putative biomarker for mood disorders. In this study however, patients with BD type I, BD type II, but not patients with SZ, were investigated. Plasma levels of circulating GDF-15 in patients relative to controls were reported without exploring correlation to psychosis severity [58]. Moreover, in the context of psychiatric illness, the association between circulating GDF-15, CRP and the influence of metabolic comorbidities is unknown.

1.5 CARDIOVASCULAR RISK IN PATIENTS WITH PSYCHOTIC DISORDER

CVD risk factors such as diabetes, intermediate stages of hyperglycemia that exist between diabetes and normal glucose homeostasis, smoking, obesity and the metabolic syndrome are common in patients with schizophrenia and other types of psychosis [59,60]. Second generation antipsychotics (SGAs) are significantly associated with weight gain, insulin resistance, diabetes, dyslipidemia and increased cardiovascular risk[61]. Furthermore, clozapine is directly associated with cardiotoxicity[62,63].

Clinical risk scores combined with biomarkers related to increased cardiovascular risk have been implemented in clinical practice in the general population. The combination has also proven useful in elderly patients[64]. Several risk scoring systems, such as the Framingham risk score (based on age, sex, smoking, diabetes, cholesterol and blood pressure levels), have been used[65]. The Reynolds Risk Score uses CRP and family history in addition to the conventional risk factors included in the Framingham risk score[66]. Studies have shown that the RRS significantly improved fit compared with either the Framingham-based Adult Treatment Panel guidelines III, CHD risk score or the newer Framingham CVD score[67].

Recent studies indicate that biomarkers reflecting blood vessel wall stress, myocardial cell damage and inflammation improve the estimate of CVD risk[68,69]. N-Terminal Pro-B-Type Natriuretic Peptide (NT-proBNP) and high-sensitive troponin T (hs-cTnT) have been shown to be useful in estimating cardiovascular risk in patients with type 2 diabetes [70]. NT-proBNP is predictive of incident CVD both in cohorts comprised of generally healthy individuals and in cohorts of high-risk individuals[71]. Hs-cTnT is specifically predictive of coronary heart disease (CHD). In the efforts made to identify markers that indicate increased CVD risk in healthy individuals, high-sensitive C-reactive protein (hsCRP) appears to be promising [72]. The use of hsCRP as a test to identify high risk patients has been evaluated in numerous studies. Among patients with non-affective psychosis hsCRP has previously been shown to be associated with increased risk of metabolic syndrome, high triglyceride levels and larger waist circumference[73].

The presence of psychotic symptoms, the core of severe mental illness, takes health care's focus away from somatic symptoms and disease. Patients with severe mental illness like schizophrenia are also less likely to seek medical care, even during acute symptoms of CHD [74]. Somatic symptoms may therefore be underdiagnosed and less monitored, potentially contributing to the increased mortality in patients with mental illnesses[75]. Broadening the focus of treatment in psychotic disorders to include somatic illness and cardiovascular risk factors with close monitoring of blood-pressure, fasting glucose and potentially the use of biomarkers might decrease the mortality.

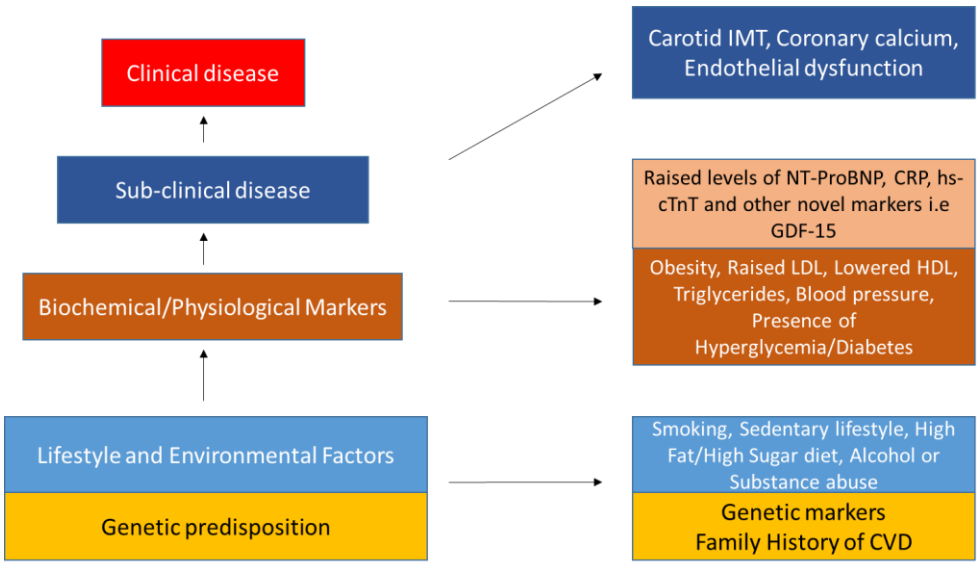

Figure 3: CVD is complex disorder which develops in the patient due to various extrinsic and intrinsic factors. Beginning with a genetic predisposition as indicated by genetic markers or a family history of CVD, the condition can be exacerbated by lifestyle and environmental factors such as smoking, a sedentary lifestyle, a high fat or sugary diet, alcohol or substance abuse. This translates to elevated CVD clinical risk markers such as the presence of obesity, raised LDL, lowered HDL, raised triglycerides, increased blood pressure, presence of diabetes or hyperglycemia along with increased levels of canonical and novel markers of CVD. In the next phase sub-clinical CVD diseases such as carotid intima-media thickening (IMT), coronary calcium aggregation and endothelial dysfunction occur. Finally these factors in combination give rise to bona-fide CVD.

1.6 PHYSICAL ACTIVITY AS AN INTERVENTION IS PSYCHOTIC DISORDER

Cognitive decline is prevalent in patients with SZ[76] and BD[77]. Physical activity as a prophylaxis against cognitive decline and dementia is well supported by individual studies and has started to gain currency in the clinic as a treatment against mild cognitive impairment. In a follow up study of more than 6000 eligible subjects aged 65 years and older, physical exercise was associated with lower risk of cognitive impairment. Additionally, there was also a lowered risk for Alzheimer's disease. Both effects were significant after being age, sex and education level adjusted[78]. In another study which looked at elderly women and the effects of physical activity on cognitive decline it was reported that after adjustment for age, educational level, comorbid conditions, smoking status, estrogen use, and functional limitation, women in the highest quartile of physical activity remained less likely than women in the lowest quartile to develop cognitive decline[79]. Clinical intervention in cases of mild cognitive impairment involve an encouragement towards lifestyle changes that include an increase in physical activity [80]. Physical activity intervention in SZ patients has been shown to improve cardio-respiratory fitness there is however little known about the effects on the cognitive function and function in daily life[81]. Exercise itself has potential benefits for those affected by psychosis, and has shown no evidence of harm in systematic reviews and meta-analyses[82,83]. Apart from the

cardiovascular benefits of exercise[84] , the anti-inflammatory effects of exercise[85] and general systemic effects of exercise[86], are well founded (Figure 4).

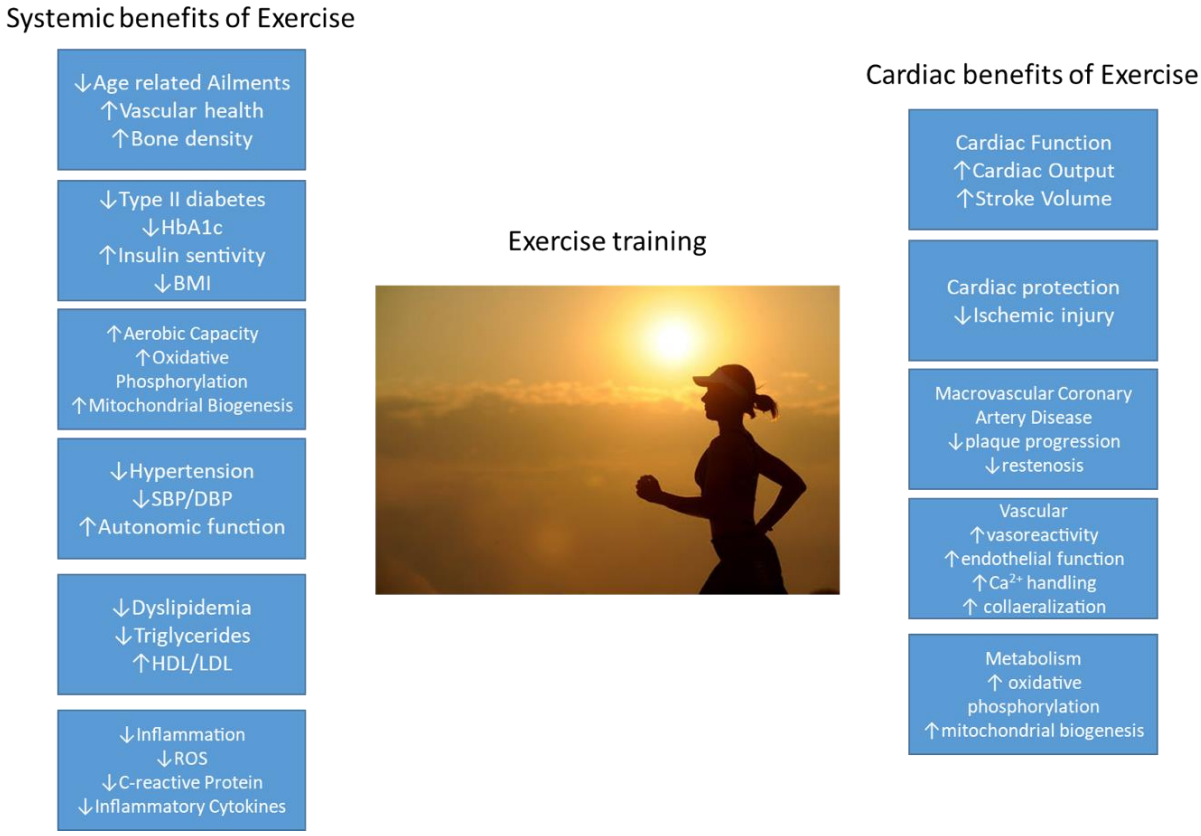

Figure 4: Physical exercise's benefits are manifold. Systemically it helps to alleviate age related ailments such as osteoarthritis, osteoporosis, dementia and maintains vascular health[87]. Physical exercise protects against Diabetes type II, lowers glycated hemoglobin (HbA1C) levels, increases insulin sensitivity[88]. Physical exercise maintains bone density, lowers BMI, increases aerobic capacity, increases oxidative phosphorylation and promotes mitochondrial biogenesis[86]. In addition to this hypertension is avoided by lowering systolic and diastolic blood pressure[89]. Through physical exercise dyslipidemia can be regulated, where triglyceride levels are reduced and HDL/LDL ratio increased[90]. Physical exercise attenuates inflammation, excessive ROS production, attenuates pro-inflammatory cytokines and reduces CRP levels[85]. Physical exercise improves cardiac function through increasing cardiac output and stroke volume[91]. Cardiac protection is afforded by physical exercise by reducing the chance for ischemic reperfusion injury[92]. Plaque progression and re-stenosis is slowed down which helps protect against macrovascular coronary artery disease[93]. Cardiovascular healthy is promoted by improved vasoreactivity, endothelial function, Ca^{2+} handling and blood vessel collateralization[94]. Cardiac metabolism is improved through better oxidative phosphorylation and mitochondrial biogenesis[95].

"A journey of a thousand miles must begin with a single step."
"Everything has its beauty, but not everyone sees it."
- Confucius

2 AIMS

The overall aim of the thesis was to identify novel molecular blood markers of psychosis.

The specific aims of each constituent study in the thesis are listed below:

2.1 STUDY I

To determine if mitochondrial DNA (mtDNA) copy number was associated to severity of psychotic illness, exposure to specific antipsychotic drugs, and degree of metabolic comorbidity in psychosis patients.

2.2 STUDY II

To determine if plasma Growth Differentiation Factor-15 (GDF-15) levels were different in psychosis patients compared to healthy controls, associated with age, severity of psychotic illness, and with degree of metabolic comorbidity and CRP levels in the patients.

2.3 STUDY III

To investigate the profile of plasma GDF-15 levels in comparison to clinical cardiovascular disease (CVD) risk factors and established CVD biomarkers and C-reactive protein.

2.4 STUDY IV

To investigate markers of immune activity in first episode psychosis (FEP) patients at baseline and changes over 12 weeks of physical exercise, and relate that to baseline clinical characteristics and amount of exercise.

3 MATERIALS AND METHODS

3.1 THE SWEDISH STUDY FOR METABOLIC RISK IN PSYCHOSIS

The Swedish study for metabolic risk in psychosis (SMRP) aimed to investigate the presence and causes of metabolic comorbidities in patients of psychosis. Participants in the studies in this thesis were recruited between 2005 and 2009 at outpatient clinics for psychosis treatment mostly within Stockholm County. Blood samples and clinical data such as body weight, height, BMI, waist circumference, fasting glucose levels, blood lipid profile, cholesterol, HDL, LDL were collected. Patients were queried on alcohol and tobacco use, previous clinical diagnoses, and psychosis severity using the Clinical Global Impression-Severity (CGI-S) index[96] functioning using Global Assessment of Functioning scale[97], general health status, family history of diabetes, medication and dosage. Medications and diagnoses were confirmed in medical records. Ethical permits for the use of clinical samples were obtained from the regional ethics review.

3.2 THE FIT FOR LIFE STUDY

The Fit for life study was conducted in Stockholm, Sweden. Patients with first episode psychosis (FEP) were approached at three psychiatric outpatient clinics specialized in the treatment of FEP in young adults, ages 18-45 years. Each clinic served approximately 200 individuals, most of whom were diagnosed with SZ. To participate, individuals had to be formally diagnosed with a psychotic disorder and receiving specialist care for FEP with at least one documented episode in the preceding five years. At the time of referral to the specialist clinic they must also have been under 40 years of age. Volunteers were excluded if they were deemed too sick to participate by the individual's clinician or had a history of CVD or physical injury that would make physical exercise unsuitable or unsafe. Patients were recruited through advertisement posters placed in common areas of the clinics. Once the individual had registered their interest in participating, their details were passed to a research nurse coordinating recruitment. Eligibility of participants was confirmed at a short interview where they were provided with information through written and verbal communication. Baseline information and written consent were then obtained. Physiological measurements were taken at baseline and within one week after the last exercise session at the Swedish School of Health and Sports Medicine (GIH) by research assistants. Each individual was requested to attend 3-5 sessions per week for the duration of the 12-week program. Each session entailed a brief warm-up lasting 5-10 minutes before an intensive 50-minute workout focusing on resistance training and cardiovascular fitness, followed by stretching, with a total duration of approximately 1 hour. Due to the nature of the patient cohort small group sessions tailored to the capability and fitness of the participants were organized. Sessions were individualized to cater to participant requirements and thus maximize attendance. Some patients expressed difficulties with specific exercise tasks, group sizes, or exercise setting. The exercise regime adhered to the American College of Sports Medicine consort agreement on prescription of exercise as an effective intervention[98]. All sessions were conducted by trained instructors at GIH being final semester students of the health coaching bachelor program conducted at GIH. This program is a three-year education in sport science, public health and includes both scientific and sports research. The Borg scale[99], an estimation of the pulse rate, was used by trainers during the session to monitor and adjust the exercise intensity for the individual. Trainers also maintained contact with participants throughout the study contacting them via SMS or phone prior to most sessions.

3.3 HEALTHY CONTROLS

In Paper II and Paper IV analyte levels in samples from psychosis patients were compared to levels in samples from healthy controls. In Paper II the healthy controls had no severe psychiatric disorder and were selected to have similar distribution of age, gender and BMI as the psychosis patients studied. All subjects had normal glucose tolerance and a family history of diabetes corresponding to the population average in Sweden. In Paper IV, the healthy controls had no psychiatric diagnosis and were selected to have similar distribution of age, gender as the psychosis patients studied. The healthy control samples had been collected and stored the same way as the psychosis samples they were compared to.

3.4 DNA EXTRACTION AND MITOCHONDRIAL DNA COPY NUMBER ANALYSIS

Venous blood was collected from patients in SMRP and DNA was extracted using a standard phenol-chloroform method [100] followed by desalting using Illustra NAP-5 columns (GE Healthcare, Buckinghamshire, UK). DNA was quantified spectrophotometrically using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Mitochondrial DNA copy number per copy of nuclear genome (i.e. per cell) was determined using real-time quantitative PCR (qPCR) as per Rooney JP[51] and Venegas V [101] protocol. Relative amount of the mitochondrial gene *tRNA-Leu*(UUR), to nuclear single-copy gene β2-microglobulin (*B2M)* (mtDNA copy number) was determined by standard curve. Briefly, each DNA sample (4.0 ng) was assayed for *tRNA-Leu*(UUR) and *B2M* within the same 384-well plate, in triplicates. DNA was amplified by using Power SYBR Green in 10 µl reaction volume, in total. The reaction was performed using QuantStudio 7 Flex (Applied Biosystems, Waltham, MA, USA). The following conditions were used: 95°C for 15 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by a dissociation stage to control for amplification specificity. Identical standard curves of control genomic human DNA (Applied Biosystems) ranging from 10 ng to 0.016 ng, was run on each plate for both genes. This was used to determine the quantity of each gene for each sample thus controlling for differing amplification efficiencies between mtDNA and *B2M*. Gene amplification product quantities were used to determine the M/S ratio for each sample. Amplicons with a Ct value outside the standard curve or a Ct standard deviation of ≥ 0.3 between triplicate or were excluded from the analyses. Patient samples were assayed in 12 consecutive qPCR plates. R^2 coefficients of standard curves were greater than 0.99 for each primer set in 384-plate. The inter-plate coefficient of variation (CV) of M/S ratio was 8.3%. This was calculated as a mean from three inter-plate control samples run in the 12, 384-well plates. The mean intra-plate CV of M/S ratio of the three control samples in triplicates was 4.6%. The mtDNA analysis detection success rate was deteremined to be a 100%. As a precaution, primer binding regions were selected for low deletional <3%[102] or mutational (SNPs) exposure 2.2%[103]. A literature and mtDNA database search revealed that no known psychiatry related SNPs are present in the primer binding mtDNA regions (Online Mendelian Inheritance in Man, OMIM®[104] and www.mitomap.org[103]). The probability of random somatic mutations occurring at the selected primer binding regions is determined to be 0.24%. For a subset of the patients an alternative amplicon in the D-loop region of the mitochondrial genome was targeted for the quantification of mtDNA for the confirmation of the sensitivity of the assay. The D-loop region was selected based on Bai and Wong [102] and was designed from mtDNA sequences that do not contain reported mutations or polymorphisms occurring in more than 1.6% of the population (http://www.genpat.uu.se/mtDB/Polysites[103,105]). The D-loop region has been used for mtDNA content analysis in similar investigations for the quantification of mtDNA copy number (cn)[106,107]. mtDNAcn measurements targeting the D-loop and *tRNA-Leu*(UUR) in the same samples were compared and found to be highly correlated, n = 55, r = 0.996, p = 1.62E-58. The primer sequences were (written 5'→3'): mtDNA Fw: CAC CCA AGA ACA GGG TTT

GT; mtDNA Rv: TGG CCA TGG GTA TGT TGT TA; *B2M* Fw: TGC TGT CTC CAT GTT TGA TGT ATC T ; *B2M* Rv: TCT CTG CTC CCC ACC TCT AAG T. Alternative primer binding region in D-loop of mitochondrial genome (written 5'→3'): D-loop Fw: CAT CTG GTT CCT ACT TCA GGG; D-loop Rv :TGA GTG GTT AAT AGG GTG ATA GA. The success rate of mtDNA copy number measurement was 97% (594/614 samples). mtDNA copy number was corrected for platelet and leukocyte count. These variables are known to affect MS ratio as platelets have mitochondria and accompanying mtDNA but no nuclei and hence lack a nuclear genome, which results in an overestimation of mtDNA[108]. Whole blood derived mtDNA are associated with leukocyte count as reported by several groups and thus a correction for the platelet and leukocyte have been suggested as a refinement to the mtDNA copy number measurement[108-110].

3.5 SINGLEPLEX AND MULTIPLEX ELISA

Plasma samples from SMRP were assayed for GDF-15 protein levels with the Quantikine® Singleplex ELISA Human GDF-15 immunoassay (R&D Systems) according to the manufacturer's instructions. A subset of the samples were assayed with technical replicates. The optical density of sample wells was determined using the Microplate Reader by Thermo Labsystemsm Inc, and the sofware Multi-skan Ascent™ for processing the data. The GDF-15 concentration of each sample was calculated using the measured absorbance against a standard curve. All samples had been freeze-thawed only once prior to analysis and all samples were run within the same week

For the purpose of tracking inflammatory profile of FEP patients in the Fit for Life study (**Paper IV**) 18 analytes (C-Reactive Protein (CRP), Eotaxin-1, Fractalkine, Gro-α, Interferon-γ (IFN-γ), interleukin (IL)-10, IL-12 subunit p40 (IL-12p40), IL-17A, IL-18, IL-1β, IL-2, IL-22, Interleukin-2 receptor subunit alpha (IL2Rα), IL-6, monocyte chemoattractant protein 1 (MCP1), tumor necrosis factor alpha (TNFα), TNF-related apoptosis-inducing ligand (TRAIL) and Vascular endothelial growth factor (VEGF) were assayed in 49 patient plasma samples drawn at baseline and follow-up and 42 healthy control plasma samples drawn at baseline (since no exercise intervention). The V-plex™ vascular injury panel 2 human kit was used to measure CRP in plasma. The U-plex™ multiplex ELISA system was used to measure Eotaxin-1, Fractalkine, Gro-α, IFN-γ, IL-10, IL-12p40, IL-17A, IL-18, IL-1β, IL-2, IL-22, IL2Rα, IL-6, MCP1, TNFα, TRAIL, VEGF. The U-plex system is limited to 10 analytes per plate and requires compatibility for the analytes to be tested in the same run to avoid antigen antibody cross-reactivity. Thus 2 plate configurations were used to cover the 17 analytes being tested. A 10-plex ELISA was used to assay Fractalkine, Gro-α, IFNγ, IL-10, IL-12p40, IL-17A, IL-1β, IL-2, IL2Rα, IL-6 and a 7-plex ELISA was used to assay Eotaxin-1, IL-18, IL-22, MCP-1, TNF-α, TRAIL and VEGF.

Both high sensitive multiplex enzyme-linked immunosorbent assay (ELISA) systems were developed by Meso Scale Diagnostics (MSD), Gaithersburg, Maryland, USA. ELISA was performed according to the manufacturer's instructions using provided instruments and the MSD software Discovery Workbench version 4.0. All plasma samples had been freeze-thawed twice. The patient plasma samples and the manufacturer-delivered standard curve samples and calibrators were measured in duplicates, and analyte-specific intraplate coefficients of variation (CVs) were determined from the standards and calibrators. Samples at baseline and follow up from each patient were measured in the same plate to reduce variability. All standard curves had a robust correlation (R^2>0.999). The healthy controls were measured in singlets as intra-plate CVs were small in all previously measured samples (<5%). Inter-plate CVs were < 13% for all analytes. Inter-plate CV for VEGF was 12.8%, IL-18 was 12.0%. Eotaxin-1, IL-17A, IL-10, IFN-γ, MCP-1, IL-22, TNF-α and TRAIL registered inter-plate CVs between 5-10%. The rest of the analytes had interplate CVs < 5%. For the purpose of statistical analysis, analytes

with signals below the level of detection (LLOD) were set to zero (concentration corresponding to blank signal) in accordance to previously published methods[111]. Data is presented as calculated mean values of undiluted plasma.

"The old pond;

A frog jumps in —

The sound of the water."

- Basho

4 RESULTS AND DISCUSSION

In this section the essence of the major results and points of discussions of the constituent papers are presented and discussed. A more detailed and comprehensive description can be found in the papers and manuscripts appended to this thesis.

4.1 MITOCHONDRIAL DNA COPY NUMBER IN PATIENTS WITH CHRONIC PSYCHOTIC DISORDER (PAPER II)

Mitochondrial DNA is vulnerable to the ubiquitous nitrosative and oxidative free radicals present within the mitochondria. Previous reports of mitochondrial dysfunction and mtDNA depletion in psychotic disorders[112], led us to the investigation of mtDNA copy number in whole blood extracted leukocyte DNA[113,114]. MtDNA copy number per cell was estimated after correction for leukocyte count and platelet count, an important consideration as (i) platelets have mitochondria and accompanying DNA but no nuclear DNA, (ii) number of platelets and leukocytes vary between individuals, and (iii) some antipsychotic treatment has been shown to be toxic to white blood cells[115]. Additionally, to account for loss of signal due to deletion events in the mtDNA[102], the selection of genes or mtDNA regions to be analyzed were based on the frequency of deletion events taking place in selected regions, obtained from mtDNA databases and existing literature, thus, regions resistant to major known deletion events were chosen[116,117]. **Paper I** is one of few studies to have taken into account the above technical considerations where mtDNA copy number research in the material of patients of psychotic disorders is concerned. A uniformly applied methodology similar to the above will ensure that individual studies may be compared to each other and represent the true quantity of mtDNA copy number in patient material.

4.1.1 Mitochondrial DNA copy number depletion is associated with the use of clozapine and risperidone

Independently of the psychosis severity of the patient, we found that the use of clozapine and risperidone amongst other anti-psychotic treatment elicited a similar depletion effect on mtDNA. The effect was dose dependent as patients' prescribed dosage of clozapine and risperidone was associated with mtDNA copy number. For clozapine, Spearman's ρ was = -0.351, $p<0.01$, n = 61 and for risperidone Spearman's ρ was = -0.233, $p<0.05$, n = 91. Interestingly for those patients on clozapine and risperidone the proportion of life on treatment was the best predictor of mtDNA copy number (β=-0.215 and $p < 0.05$) This further confirms the distinct effect of clozapine and risperidone we saw on mtDNA copy number in our patient cohort. In our analysis we had an acceptable statistical power to detect an effect of other anti-psychotic drugs where patient cohorts were larger than 40, such as olanzapine n=108 and aripiprazole n=73 but no such effect was found.

For patients on other types of anti-psychotic treatment, age and psychosis severity were the best predictors of mtDNA (β_{CGI-S}=-0.129 and $p < 0.05$, β_{Age}=-0.159 and $p < 0.01$). For psychosis severity estimation we relied on the routinely used the CGI-S index, which was adapted to be used in the setting of psychosis outpatient clinics. This symptom based adaptation of CGI-S is possible as the clinicians are encouraged to use the patient cohort as a reference group[96]. In the context of psychosis CGI-S has shown to have considerable overlap with severity scales which are tailored for psychotic disorders such as SZ[118].

4.1.2 Mitochondrial DNA copy number reduction in human neurons

A further step we took in **Paper I** was to assess whether the effect of clozapine and risperidone found on mtDNA copy number in whole blood also was detectable in human neurons from NESCs. The neurons were established to have similar morphological and electrophysiological characteristics as human neurons *in vivo* [119,120]. Human neurons when treated in cell culture medium with pharmacological doses of clozapine simulating target CSF levels exhibited a 16% reduction in mtDNA copy number compared to vehicle treated cells ($p<0.01$). Human neurons which were treated with risperidone at target CSF levels did not experience any effect. However, at target plasma concentrations, there was an estimated 14% reduction in mtDNA copy number in the human neurons exposed to risperidone and 25 % reduction in mtDNA copy number for human neurons exposed to clozapine.

4.2 PLASMA GDF-15 LEVELS ARE ELEVATED IN PATIENTS OF CHRONIC PSYCHOTIC DISORDER (PAPERS II AND III)

Currently, elevated plasma GDF-15 levels has been reported in a CVD and metabolic disorders[121,122]. In the context of unipolar depression, depression with BD I and BD II, it has been reported that GDF-15 levels are statistically different between the groups[58], however much less information is available of GDF-15 in the context of psychotic disorders. In paper II we analyzed patient plasma for GDF-15 in n=120 patients of psychotic disorder and made a comparison to age and gender matched healthy controls. We found that there was a significant elevation in plasma GDF-15 levels in patients, median of patients=744 ng/mL, median of controls=516 ng/mL, $p<0.001$. We further analyzed GDF-15 for association to several clinical variables which reflected the severity of the psychosis, metabolic comorbidity and lifestyle factors and found GDF-15 to be associated with gender and smoking, where men had increased levels of GDF-15 ($p<0.05$) and smoking was associated with increased plasma GDF-15 levels ($p<0.01$).

4.2.1 GDF-15 is robustly correlated with age in patients of chronic psychotic disorder (Paper II)

GDF-15 was robustly associated with age in the patients (Spearman's $\rho = 0.496$, $\beta_{Standardized} = 0.494$, $p < 0.001$). GDF-15 has been reported to be an all-cause mortality marker[123] and well described for age dependent reference limits with prognostic relevance[124]. Confirming that such a robust association exists with age in the presence of psychotic disorder is important information for assessing the usefulness of GDF-15 as a biomarker in the context of psychotic disorders. The presence of age dependent reference limits opens up the possibility for testing the biological age of patients of psychotic disorders when taken together with other biomarkers.

4.2.2 GDF-15 levels are associated with psychosis severity as measured by CGI-S

Psychosis severity as measured by CGI-S was negatively associated with GDF-15 levels ($\beta_{standardized} = -0.221$, $t = -2.58$, $p = 0.012$). This observation resonates with the anti-inflammatory function of GDF-15. A growing body of evidence suggests that inflammation is involved in the aetiology of psychosis[32,125-128]. Thus, the depletion of anti-inflammatory agents such as GDF-15 may lead to more severe psychosis symptoms or poorer functionality.

4.2.3 Cardiac biomarkers for patients with chronic psychosis (Paper III)

Description of CVD risk markers in a patient cohort of psychotic disorder reveals a latent risk of CVD. The elevated risk of CVD in patients of psychosis has been well established and is often the cited reason for early mortality in these patients[61,74,129]. In mental health disorders such as psychotic disorders, the focus on treating the psychiatric symptoms often takes away the attention from the potential somatic comorbidities such as metabolic syndrome and CVD arising from genetic and environmental factors. Thus, in **Paper III** we sought to address the gap in CVD risk monitoring in psychosis patients using the SMRP patient cohort by investigating levels of three established CVD biomarkers as well as the putative CVD risk marker, GDF-15, in relation to frequency of elevated levels and association to other established clinical markers of CVD risk. We show that our study cohort of patients with psychotic disorder, similarly to other psychosis cohorts, had an elevated risk for CVD as 36% had clinically elevated hs-CRP levels, 8% had pathological hs-cTnT levels, and 7% had pathological NT-proBNP levels, indicating latent and undiagnosed CVD. Also, based on published healthy control reference values[124] we saw that 25% of the cohort had elevated GDF-15 values.

4.2.4 GDF-15 resembles more closely a cardiac biomarker than acute inflammation

In bivariate correlation analyses we saw that GDF-15 was correlated with both NT-proBNP (Kendall's tau-b=0.171, $p<0.01$) and hs-cTnT levels (Kendall's tau-b = 0.312, $p<0.01$), but not with hs-CRP. This could be an indication of GDF-15 to more closely resemble a cardiac function marker than a marker of inflammation such as hs-CRP. However, GDF-15 levels were not associated with clinical CVD risk factors including descriptors of metabolic health, such as, systolic pressure, blood lipids, fasting glucose or waist circumference. Variance of hs-CRP, NT-proBNP and hs-cTNT levels as dependent variables in linear regression analysis were better explained by metabolic health descriptors than was the variance in GDF-15 levels. However, the GDF-15 level variance could be best explained of the four, due to the robust dependence on age, which in itself is an interesting observation. As observed in **Paper II** GDF-15 levels were elevated in patients of psychotic disorders compared to age and gender matched healthy controls, while in **Paper III** the data propose that this elevation might be explained by latent CVD as GDF-15 levels which correlated well with hs-cTnT levels[130], which is reflected as accelerated ageing *in vivo* by plasma GDF-15.

4.3 EXERCISE INTERVENTION IMPROVES FITNESS AND INFLAMMATION PROFILE IN FIRST EPISODE PSYCHOSIS PATIENTS

Paper IV was a study which took place in the context of the "Fit for Life" study[131-133] which aims to complement clinical treatment of psychosis with exercise intervention, with a particular focus on mitigating the ill effects of a sedentary lifestyle[134] and social isolation that patients of psychosis often experience[135]. As a result of the intervention patients' experienced improved levels of cardiorespiratory fitness, 27% of the cohort attained the VO_2 max level prescribed for their age and gender. In addition to this, CRP was reduced over the intervention (Mean ΔlnCRP = -0.36, SD ΔlnCRP = 1.04, t = -2.37, p = 0.02), although still elevated post-intervention compared to healthy controls ($p<0.01$).

4.3.1 Fractalkine levels are elevated in First Episode Psychosis

In paper IV, we made a novel observation that plasma soluble fractalkine levels were elevated in patients with FEP when compared to age and gender matched healthy controls. Apart from general improvements in fitness and inflammation profile, reduction in fractalkine levels in the patient cohort can be viewed as a clinical gain for the patients undergoing the above intervention, when considering fractalkine's role in inflammation. Fractalkine is a ligand for CX3CR1 and integrins[136]. Membrane bound fractalkine is cleaved by metalloproteinases, induced by TNFα, into a soluble ligand form of fractalkine[137] which is chemotactic to cellular mediators of immunity such as T-cells, microglia and monocytes[138]. CX3CR1 the cognate receptor of fractalkine is expressed by microglia, potentially to receive activating signals in the form of soluble fractalkine released by neurons (Figure 5). Systemically fractalkine is expressed by vascular endothelial cells induced by a Th1 cytokine profile[137,138] which allows cellular mediators of immunity to extravasate between vascular endothelial cells along a chemokine gradient to be recruited to sites of inflammation.

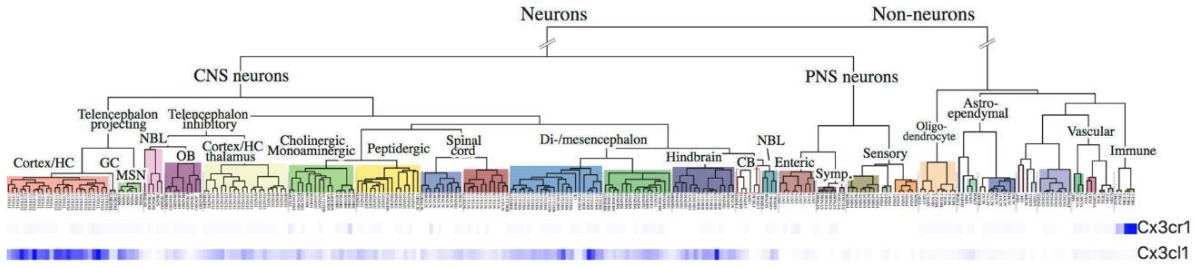

Figure 5: Cell types in regions of CNS and PNS of mice indicated and grouped as a tree diagram. Expression of fractalkine (CX3CL1) is indicated by lower blue bar, present across most neuronal cell types, especially those localized in the cortex, hippocampus and di-/mesencephalon. Fractalkine expression is absent immune cells. Conversely, the cognate receptor for fractalkine CX3CR1 expression is absent in neuronal subsets and richly present in immune cells. Neuronal signaling and activation of microglia could be regulated by fractalkine. Graphic generated on http://mousebrain.org/genesearch.html..

Raw data available on https://www.ncbi.nlm.nih.gov/sra/SRP135960

4.3.2 Subgroups defined by changes in fractalkine

The present study showed that plasma fractalkine is significantly elevated at group level in those with FEP compared to healthy controls ($p<0.01$). While 34.7% of the FEP were at fractalkine level seen in healthy controls, the remaining 69.4% (n=34) had fractalkine levels far above those reported for sepsis and after induction by intravenous injection with lipopolysaccharide (4 ng/kg)[139]. The FEP patients were found to further categorize into fractalkine level-defined groups by exploring the response to structured exercise for 12 weeks. While all FEP patients grouped together showed a reduction in plasma fractalkine by the intervention (median ΔsFKN, -295 pg mL^{-1}), $p<0.05$, repeated measures ANOVA (RMA)), reflecting a potential anti-inflammatory effect of physical exercise[140] , four fractalkine response groups were identified where the fractalkine level could be represented by either 'on' or 'off' at baseline/follow-up: stably low fractalkine levels (off/off, n=12), responders (on/off, n=20), non-responders (on/on, n=12) and reverse responders (off/on, n=5).

4.3.3 Those with reduced fractalkine levels after exercise intervention had less severe psychotic disorder at baseline

Fractalkine response was tested for association with baseline clinical parameters and drug treatment. Fractalkine responders had a shorter time since first psychiatric contact ($\chi2 = 11.83$ (3 df) $p = 0.008$). Accordingly, time since first psychiatric contact associated with change in plasma fractalkine level also when adjusting for sex, age and use of antipsychotic drug [yes/no] ($p=0.02$). ASD and ADHD comorbidity was present in 26.2% of patients and was more common among fractalkine responders 43.8%. Reduction in plasma fractalkine was greater among participants with ADHD/ASD comorbidity ($U=2.39$, $p=0.016$). Psychosis severity in these patients is often over-estimated due to the presence of ADHD/ASD. Taking the reduced duration of psychiatric syndrome and presence of ADHD/ASD as indicators of less severe psychotic disorders we reasoned that the fractalkine response to exercise intervention was a result of less severe profile in the patients at baseline.

4.3.4 Those with high fractalkine level before and/or after exercise had an altered immune activity profile at baseline

IL-12, Eotaxin-1, VEGF, IL-17A, TRAIL and IFN-γ had differing baseline levels in patients when they were grouped as fractalkine response groups versus healthy controls, with the exception of the "stably low" group of patients. The "stably low" group of patients had a similar profile to the healthy controls of the study, while the other groups, responders, non-responders and reverse responders had similar immune activity profiles. IL-12 levels were elevated in patients ($p<0.01$) compared to controls while stably low sub-group of patients had lower levels of IL-12 than the responders ($p<0.01$) and non-responders ($p<0.01$). Patients as a whole had lower levels of Eotaxin-1 compared to healthy controls and in addition responders had lower levels of Eotaxin-1 compared to the stably low patient group. VEGF levels were higher in healthy controls compared to patients ($p<0.01$). VEGF levels were higher in responders ($p<0.01$) and reverse responders ($p=0.03$) compared to non-responders. IL-17A levels were higher in patients as a whole compared to healthy controls ($p<0.01$), and the stably low group had lower levels of IL-17A compared to the responders ($p<0.01$), non-responders ($p<0.01$) and reverse responders ($p=0.01$). Interestingly, a dysregulation of all of the above-mentioned cytokines have been reported in patient cohorts of psychotic disorders such as schizophrenia [27-32]. Comparable to our observation of reduced levels of IFNγ in the plasma, the authors Freudenreich et. al, have observed that cellular immunity is impaired in patients of

schizophrenia compared to healthy controls when PBMCs were analyzed for mRNA level expression of IFNγ[141]. Baseline levels of IL-12, Eotaxin-1, VEGF, IL-17A, IFN-γ and TRAIL showed differences between the fractalkine response groups, such that the stably low group had a pattern at baseline resembling healthy controls, while the other groups, each with high fractalkine level at least once, were at baseline indistinguishable from each other displaying elevated IL-12 and IL-17A and reduced Eotaxin-1, VEGF, TRAIL and IFN-γ levels, a cytokine profile indicating a dysregulation of the baseline immune activity in these 37 FEP patients (76% of the studied cohort). Similar to IL-12 and IL-17A, TRAIL levels were higher in healthy controls compared to patients as a whole ($p<0.01$). The stably low group of patients were comparable to healthy controls and had higher TRAIL levels than the group of responders ($p=0.028$). TRAIL has been linked with the ongoing pathology of schizophrenia and psychosis[142]. TRAIL, a relatively recently described protein ligand mediating apoptosis and member of the Tumor Necrosis Factor (TNF) family[143] of proteins, has little relevant literature in the field of schizophrenia and psychosis. Nonetheless our findings resonate with the growing understanding that TRAIL together with TNF-related weak inducer of apoptosis (TWEAK), another recently described member of the TNF family, have salient roles in the progression of neuro-inflammation in patients of schizophrenia and psychosis[142]. Interestingly we see reduced levels of cytokines i.e. IFNγ and TRAIL, in the same patients who fall in the category of high fractalkine at one or both of the timepoints measured, compared to healthy controls. This could be a result of the feedback which is necessitated by high levels of fractalkine in plasma, as IFNγ and TNFα are part of the battery of cytokines which promotes the release of membrane bound fractalkine into circulation. Taken together this puts forth the case for further research into fractalkine as a marker for ongoing inflammation in patients with psychosis.

4.3.5 The fractalkine response groups were distinct in Gro-α and IL-18 changes.

The three fractalkine response groups with apparently similarly altered baseline immune activity could be differentiated by studying the change in Gro-α and IL-18 levels over the exercise intervention. Gro-α levels increased over exercise intervention in the non-responders and reverse responders compared to responders and the stably low group ($p<0.01$). There was a trend for IL-18 levels to increase in the reverse responders group compared to the responders. Thus, Gro-α and IL-18 level change over intervention correlated highly to fractalkine level change over intervention and thereby demarcated the groups responders, non-responders and reverse responders from each other, supporting that the dysregulated immune activity in these groups could be modulated by physical exercise. Interestingly, and in parallel to our finding that Gro-α levels are distinctly different between the response groups it has been reported that Gro-α is dysregulated in FEP patients who suffered structural changes in brain such as white matter pathology[32]. Comparable to our finding that IL-18 levels are highest in the *'reverse responder'* group compared to the 'stably low' and healthy controls and other patients, it has been shown that IL-18 is increased in the plasma of FEP compared to healthy controls and is correlated with cognitive dysfunction[144].

5 SUMMARY AND CONCLUDING REMARKS

Here, the constituent papers are summarized with some concluding and general remarks. The constituent papers belong to larger projects which are ongoing and in search of better diagnostic tools and therapeutic options for psychotic disorders. Investigations in independent cohorts and replications are warranted and humbly requested.

- In **Paper I** we investigated the amount of DNA in mitochondria which is a proxy for mitochondrial biogenesis and functionality, for association with psychosis severity and treatment of psychotic disorders. We confirmed earlier reports that mtDNA copy number was associated with age. We investigated mtDNA in peripheral tissue (leukocytes) and CNS tissue (cultured human neurons) and saw that certain types of anti-psychotic treatment can be deleterious to mtDNA in both tissues. Secondary to treatment effects we saw that mtDNA copy number was associated with psychosis severity as measured by CGI-S index.
- As indicated in **Paper II and III,** patients with psychotic disorder such as, schizophrenia, are pre-disposed to metabolic comorbidities and thus shorter life span. In such patient cohorts, it could be useful to determine the accelerated ageing through analysis of the blood. We found elevated levels of GDF-15, an analyte previously shown to be associated with increased age, CVD and diabetes. Our findings point us to plasma GDF-15 as a potential tool in diagnosing accelerated ageing in patients of psychotic disorder.
- As a follow up to **Paper II, in Paper III** we asked the question if increase in plasma GDF-15 level was associated with latent CVD in a patient cohort of psychotic disorders. We found that the GDF-15 levels correlated with levels of two established analyte biomarkers of CVD but was not associated with an indicator of acute or chronic inflammation, CRP.
- In **Paper IV**, we investigated exercise intervention in patients of FEP, as an adjunct therapy. Of the analytes we measured based on an inflammation related hypothesis fractalkine levels were attenuated through exercise intervention. Interestingly response to intervention as defined by changes in fractalkine levels helped us meaningfully group the patient cohort from an inflammation biology point of view. The sub-groups had differing clinical profiles at baseline where we saw that a group of patients had a similar inflammation profile to healthy controls.

6 FUTURE PERSPECTIVES

This thesis is intended to summarize the knowledge we have gained from analyzing material obtained from patients with psychosis for the goal of improved diagnostic tools and therapeutic options available to said patients. With that goal in mind, I would like to extend a few perspectives for the future, on work that should be carried out to build on our findings for eventually clinical applications.

1. Our findings in the plasma represent information mainly centered on the systemic circulation of patients. While it is a strength that our analysis and work were conducted wholly in human material, thus having relevance for improving our understanding of human biology, schizophrenia and other psychotic disorders are disorders primarily of the CNS. As the CNS is fed by CSF that we know is relatively isolated from the systemic circulation system by the blood brain barrier, the thesis would benefit from having information on the constituent molecules of the CSF, in particular those described in more detail in the thesis, GDF-15, mtDNA and fractalkine and how well CSF values correlate with those measures in plasma. The blood brain barrier has been shown to be compromised in at least some kinds of psychotic disorders[145] which adds credence to the theory that alterations in the inflammatory profile of the systemic circulation may have an impact on the CSF[16].

2. In **Paper II and Paper III** we reported that GDF-15 levels are associated with age in psychosis patients, which has been reported previously in individuals without severe mental illness [123]. This information could be potentially used in disease monitoring, especially relevant in patients with psychotic disorders as they are prone to accelerated ageing and shorter life span. However, in **Paper II** we noted that GDF-15 levels were inversely associated with psychosis severity, that is, with increasing psychosis severity the levels of GDF-15 receded. Without a longitudinal design, it is difficult to estimate the relevance of GDF-15 as an all-cause mortality marker or an indicator of biological ageing. Currently it is understood that GDF-15 is secreted in low concentrations by most tissues, with defined roles in biological processes such as inflammation and apoptosis[146]. Interestingly it is upregulated in tissue injury, CVD and carcinogenesis[33]. However since little is known about the kinetics of plasma GDF-15 in the various pathological contexts[147], the role of GDF-15 as a biomarker in those scenarios cannot yet be evaluated. Therefore more research is required into the expression, clearance and changes in the kinetics of plasma GDF-15.

3. In **Paper III** we attempted to cast further light on the findings of **Paper II**. The increased levels of GDF-15 in the plasma of our study cohort of patients with psychotic disorder may well be due to multiple causes, in line with the literature describing GDF-15 as an all-cause mortality marker[123]. In this study we compared the levels of GDF-15 to levels of hs-CRP, NT-ProBNP and hs-cTnT, in order to evaluate whether GDF-15 levels reflect CVD pathology. Indeed we saw that there were correlations with the CVD biomarkers (NT-ProBNP, hs-cTnT), but not with a marker of acute or general inflammation, CRP[148]. We expect GDF-15 levels to be associated with other categories of biomarkers relevant to CVD, such as, coagulation, vascular function, oxidative stress, structural and functional markers relating to atherosclerosis.

4. In **Paper I** we investigated the amount of mtDNA in whole blood as a marker of ageing, psychosis severity and anti-psychotic treatment. We saw that mtDNA copy number was reduced by certain types of antipsychotic treatment, both in wole blood and human neurons. Independently of this mtDNA was also reduced with age, and increasing psychosis severity The above study would benefit from a detailed analysis of the kinetics of mitochondrial fusion and fission as it is known that mitochondria conduct the production of ATP in dynamic and complex intracellular networks[149]. The changes in mtDNA as mitochondrion shift from discrete organelles to vesicular networks and back, through a process of fission and fusion, is poorly understood, and needs to be investigated further *in vitro*¸ with respiration and functionality measures of OXPHOS using Seahorse XF Cell Mito Stress Test or OxyGraph, in parallel to studies similar to ours, [106,114] which track quantitative changes in mtDNA in cohorts of psychiatric patients.

5. In **Paper IV** we observed that fractalkine, a chemokine mediator of neuroinflammation and migration of cellular mediators of immunity is dysregulated in some groups of FEP patients. We noted that overall as a cohort, the patients had elevated levels of fractalkine, however one sub-group of patients who had lowered levels of fractalkine, had an inflammation profile which more closely resembled the healthy controls than other patients who had registered high levels of fractalkine at one of the time points measured. In future studies of psychotic cohorts, investigators may frame a study that is dedicated to exploring all aspects of fractalkine biology hitherto known. This may include selecting the cytokines which induce and inhibit ftractalkine expression and release. In addition this study can be reproduced in psychosis relevant models, *in vitro and in vivo.*

6. The projects outlined in this thesis aimed to provide pre-clinical evidence and basic research to back the use of novel comparisons between healthy individuals and patients of psychotic disorders, as markers of health, pathogenic process or pharmacologic response to therapy. With the findings of this project we hope to bring our novel observations to the threshold of basic research and one step closer to being considered as exploratory biomarkers. For this to occur, independent confirmation of our findings and more mechanistic substantiation of observations to establish casuality will be necessary. Therefore a limitation of this thesis, is that none of our observations can be considered grounds for the use of observed differences between patient and healthy controls as biomarkers, and a future direction for developing the project would be to take the necessary measures as outlined by regional Food and Drug administration bodies[150,151]

"Do the difficult things while they are easy and do the great things while they are small."

Confucius

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"It does not matter how slowly you go so long as you do not stop.

Our greatest glory is not in never falling, but in rising every time we fall.

When you are content to be simply yourself and don't compare or compete, everybody will respect you.

When I let go of what I am, I become what I might be."

- Confucius

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